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The rate of lateral diffusion of phospholipids in erythrocyte microvesicles

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^{31}P-NMR spectra of phospholipids in membranes of erythrocyte microvesicles isolated from outdated blood units were recorded in the temperature range 5 to 55°C. Within that range the lineshape is strongly influenced by an increasing rate of lateral diffusion of phospholipids. At 36°C a diffusion constant, *D*, of $(2 \pm 1) \cdot 10^{-12}$ m^2/s was obtained. The diffusion rate is by a factor of 3 to 10 greater than in erythrocyte membranes measured by the photobleaching technique and is comparable with values obtained for several lipid model membranes. The differences in lateral diffusion rates are probably connected with the depletion of microvesicle membranes in membrane proteins.

Introduction

Human erythrocytes shed microvesicles in several stress situations [1–7] and during aging in vitro, including blood bank storage [7–12]. Microvesicles from banked blood samples are spherically shaped and have rather uniform diameters of about 150 nm [9,11]. Their lipid composition is nearly identical to that of the erythrocyte membrane [8]. In comparison to the intact erythrocyte membrane, these vesicles are characterized by (i) depletion in the membrane skeletal proteins, mainly spectrin [10,13], (ii) decrease in transmembrane proteins such as band 3 protein, glycophorin, etc. [14], (iii) formation of clusters of intramembraneous particles consisting mainly of band 3 protein [11], and (iv) a more random distribution of different phospholipids between the inner and outer leaflet of the membrane [15,16]. The latter was not detected in microvesicles released from fresh erythrocytes by treatment with Ca^{2+} and A23187 [17].

The rate of lateral diffusion of fluorescence labels modelling the diffusion of lipid molecules in erythrocyte membranes was extensively investigated by the photobleaching technique [18–20]. The experimentally obtained diffusion rates are up to one order of magnitude smaller than the corresponding diffusion rates of lipids in pure lipid-water dispersions [18,21–23]. The differences in the diffusion constants were explained in terms of a mosaic-like distribution of fluid lipid regions and of rigidified lipid-protein assemblies [18]. The diffusion rate of membrane protein (band 3 protein and glycophorin) in erythrocytes labelled with fluorescence markers and measured with the photobleaching technique is indeed significantly lower than that of the lipids [20]. Because of the above-mentioned changes in the membrane protein composition, it would be of interest to measure the rate of lateral lipid diffusion in microvesicles. The diameter of these vesicles is too small for photobleaching measurements. In the present study the rate of lateral diffusion is estimated by its in-

fluence on the ^{31}P-NMR lineshape of membrane phospholipids as was done for phosphatidylcholine vesicles [24] and hepatic microsomes [25].

Materials and Methods

The vesicles were isolated and purified by a cycle of graded centrifugation steps from the spontaneous sediments of ACD-AG blood samples banked for 6 weeks [26]. To remove soluble phosphorus-containing compounds they were dialyzed for 5 days at 5°C against physiologic saline under sterile conditions with daily changing of the medium. Only those vesicle preparations were used which were free of contaminating ghost material (absence of spectrin bands after SDS-polyacrylamide gel electrophoresis and electronmicroscopic proof; see Ref. 13). For ^{31}P-NMR investigations the vesicles were suspended in physiologic saline to about 85 mg vesicle haemoglobin per ml, corresponding to 1.5 mg lipid-bound phosphorus per ml [13]. 1 ml of the sample was filled in a 10 mm sample tube. 100 μl ^2H_2O were added for ^{2}H-NMR stabilization. ^{31}P-NMR spectra were recorded on a Bruker HX-90 spectrometer at 36.4 MHz using intensive proton noise decoupling. Usually 4000 scans (flip angle, 60°; pulse repetition rate, 0.75 s) were accumulated. For proof of purity of the vesicle preparations and for estimation of the mean diameter of the vesicles they were investigated electronmicroscopically after fixation for 30 min in 0.05 M cacodylate buffer (pH 7.4) containing 4 wt% formol and 2 wt% glutaraldehyde, followed by postfixation for 60 min in the same buffer containing 1.5 wt% OsO_4 and 2.5 wt% $K_4(Fe(CN)_6)$, dehydration using acetone and propylenoxide and embedding in Durcupan ACM (Fluka, Switzerland). Only the diameters of vesicles with sharp demarcations were measured (200 vesicles per preparation).

Results and Discussion

^{31}P-NMR investigations were restricted to pure vesicle preparations (see above) with a narrow distribution of vesicle diameters. In Fig. 1 a typical electronmicroscope picture of a microvesicle preparation is given. The microvesicles are spherical; their mean diameter is 144 nm, with a root-mean-square fluctuation of 21 nm. Contamination with erythrocytes, erythrocyte ghosts and debris of the white cell lineage is not visible. At concentrations used for ^{31}P-NMR investigations most of the vesicles are aggregated. The aggregation can be revoked by dilution of the sample with physiologic saline and gentle vortexing.

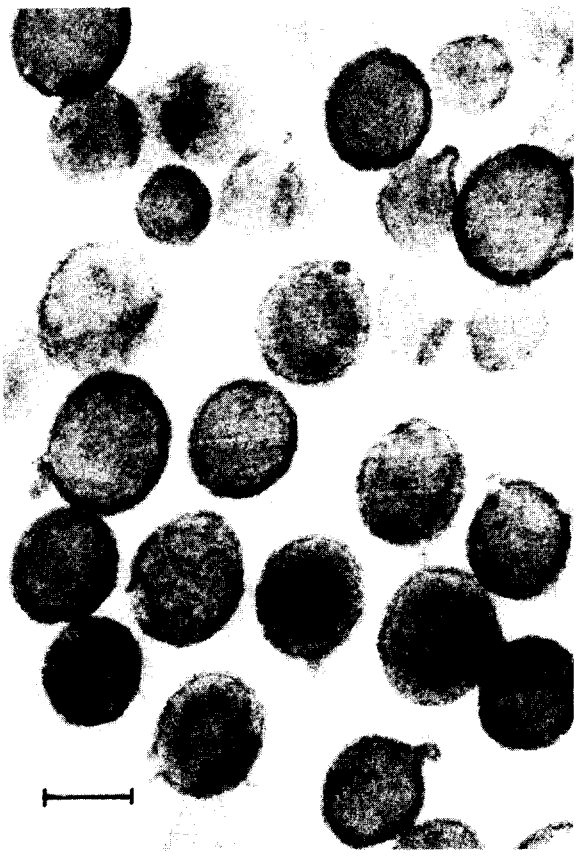

Fig. 1. Electromicroscope picture of a microvesicle preparation. The bar indicates 100 nm.

In Fig. 2 the measured ^{31}P-NMR spectra of the same microvesicle preparation are given in dependence on temperature. Below 25°C the NMR spectra are typical of a lamellar arrangement of membrane phospholipids [27]. Similar ^{31}P-NMR spectra were reported for erythrocyte ghost membranes [27,28]. At 5°C the chemical shift anisotropy ($\Delta\sigma_{eff}$) of the ^{31}P-NMR signal of microvesicles is about 40 ppm. Up to 25°C no significant changes in the spectra occur. Between 25°C and 55°C the chemical shift anisotropy averages out. During the cooling cycle the same spec-

tra are obtained, indicating that no change in the shape and size of microvesicles occurs during heating. The measurements were repeated with four independent preparations of microvesicles. Similar lineshapes were always obtained at corresponding temperatures.

During diffusion of lipid molecules over spherically shaped surfaces the orientation of the molecules to the outer magnetic field changes. If the diffusion rate is high enough and the diameters of spherical particles small enough, the ^{31}P-NMR chemical shift anisotropy is averaged out. Burnell et al. [24] monitored these changes by measuring the ^{31}P-NMR spectra of phosphatidylcholine liposomes at different temperatures. If the rate of lateral diffusion of phospholipids in the microvesicles is comparable with that of the model membrane, the lineshape of the microvesicles (d = 144 nm) should be influenced by lateral diffusion. Further changes could be caused by rotational diffusion of the whole microvesicles and by changes in the local mobility and conformation of lipid molecules. Rotational diffusion can be slowed down by increasing viscosity of the medium and aggregation of vesicles. The viscosity of the ^{31}P-NMR samples is comparable to that of a thick oil. Electron microscopy shows that most of the vesicles are aggregated. A further proof for the influence of rotational diffusion is the addition of large amounts of glycerol to the vesicle dispersion. Unfortunately, after addition of glycerol and intensive vortexing it was not possible to obtain a homogeneous solution. Because of the obvious high viscosity of the samples without glycerol and the aggregation of vesicles, the influence of rotational diffusion on the ^{31}P-NMR lineshape was assumed to be negligible. The presence of a phase transition around 20°C, mainly in cholesterol depleted erythrocyte membranes, was suggested by some authors [28–34], but drastic changes in the local mobility and conformation of the same phospholipids in erythrocytes were not detected, neither with deuterated fatty acids [35] nor with deuterated phosphatidylcholine [36] incorporated into erythrocyte membranes. Erythrocyte ghosts membranes do not show similar lineshape changes in the same temperature region [27,32]. Therefore, it is reasonable to assume that the changes are caused by increasing rates of lateral diffusion of lipid molecules over the surface of microvesicles.

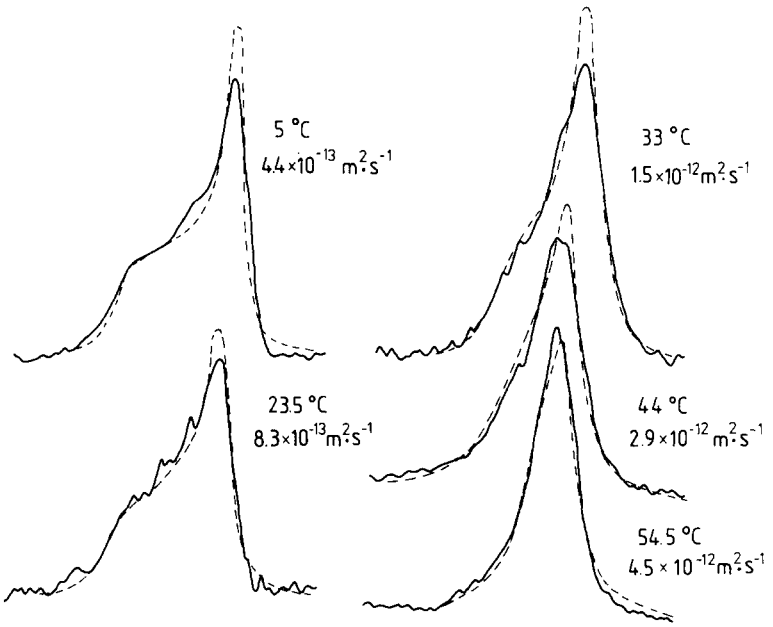

Fig. 2. Measured (———) and calculated (- - - - -) ^{31}P-NMR spectra of a microvesicle preparation at different temperatures. For the calculation procedure, a value of $\Delta\sigma_{eff}$ = 40 ppm, a resonance frequency of 36.4 MHz, a microvesicle diameter of 144 nm and the lateral diffusion constants given in the figure were assumed.

In Fig. 2 calculated ^{31}P-NMR spectra considering different lateral diffusion rates of phospholipids ($\Delta\sigma_{eff} = 40$ ppm, $d = 144$ nm) are given. Our calculation is based on the stochastic linewidth theory of Anderson and Weiss [37,38], but similar results can be obtained by the stochastic Liouville theory [39,24]. From the comparison of calculated and experimental spectra, lateral diffusion rates can be derived. Because of distortions in the experimental spectra caused by the presence of different phospholipids with probably slightly different $\Delta\sigma_{eff}$ values and by technical difficulties, a perfect fitting of experimental spectra is not possible.

Conclusions

At physiological temperature the constant of lateral diffusion is $(2 \pm 1) \cdot 10^{-12}$ m^2/s. This value is in good agreement with diffusion constants of lipid model membranes [18,21–23] and with local lipid diffusion constants in erythrocytes obtained with the fluorescence excimer formation technique [18]. It is by a factor of 3 to 10 greater than the constant of lateral diffusion over larger distances measured using the photobleaching technique in erythrocytes [18–20]. In our ^{31}P-NMR experiments, diffusion is measured as a change of the orientation of the lipid molecules to the outer magnetic field over an angle of $\pi/2$. This corresponds to a diffusion pathway of approx. 110 nm, which is closer to the diameter of a laser spot in photobleaching experiments than to the characteristic length of a pathway in fluorescence excimer formation. The difference in lateral diffusion between erythrocytes and microvesicles can be explained probably by the lower membrane protein content and by the more random distribution of phospholipids in the microvesicles in comparison to intact erythrocyte membranes. The existence of rigidified lipid-protein assemblies introduced for the interpretation of experimental results with erythrocyte membranes could not be established for microvesicles formed during banking of erythrocytes.

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